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Resistance in Breast Cancer Patients

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**MAIN REPORT**

**ABSTRACT**

The non-steroidal antiestrogen tamoxifen (TAM) has been used successfully in the treatment of tens of thousands of women with breast cancer, and has been shown to increase both disease-free and overall survival in treated patients. Unfortunately, virtually all patients treated with TAM eventually develop resistant disease. The research funded by this fellowship is directed at increasing our understanding of the mechanisms leading to the development of tamoxifen resistance.

Specific Aim 1 of this proposal has been completed and three altered estrogen receptors (ERs) have been identified from clinical samples, one of which exhibits a hormone-independent phenotype in transient assays. We are currently generating the necessary constructs for screening this ER variant for activity consistent with the development of hormone-independent growth *in vivo* (Aim 2). We have also worked out the technical aspects of microdissection of archival and frozen clinical tumor samples to improve the use of differential display technologies to examine these samples for altered gene expression coincident with the phenotype of tamoxifen resistance (Aims 3 and 4).

**INTRODUCTION**

Breast cancer is the most common cancer among women in America, and is second only to lung cancer as the cause of cancer death. Indeed, this year alone almost 180,000 American women will be diagnosed with the disease. Fortunately, many of these women will have tumors that are dependent upon estrogen for their growth, and these patients will be successfully treated with endocrine therapies which are associated with much less severe side-effects than most forms of chemotherapeutic treatment. The antiestrogen tamoxifen (Tam) is the most successful of these endocrine treatments, and has been shown to prolong

disease-free and overall survival both in cases of advanced disease (1, 2), and when used as an adjuvant treatment (3). Unfortunately, however, most patients who are successfully treated with Tam eventually fail therapy, and their disease progresses to become Tam resistant.

Studies designed to increase our understanding of the mechanisms leading to Tam resistance are the subject of this postdoctoral fellowship proposal. In order to better understand the phenomena contributing to Tam resistance, we proposed the following Specific Aims:

1. To investigate the association between clinical Tam resistance and the presence of altered ERs.
2. To evaluate the functional activity of any receptor variants identified in Aim 1.
3. To identify new genes using differential display, the expression of which is associated with clinical Tam resistance.
4. To confirm the identify and evaluate the function of any genes identified in Aim 3.

## **BODY:**

### **Specific Aim 1**

Thirty metastatic Tam-resistant breast cancer samples were screened for mutations in the ER gene by SSCP analysis. SSCP shifts (evident by electrophoresis both at ambient and subambient temperatures) were identified in six of the metastatic tumors. The six positive samples were further studied by both direct sequencing analysis, and cloning of shifted bands followed by sequencing analysis. Three of the SSCP shifted bands were found to constitute previously identified polymorphisms: TCT to TCC at nucleotide 30 in

exon 1, TGC to TGT at nucleotide 720 in exon 3, and CGC to CGT at nucleotide 729 in exon 3, none of which results in amino acid substitutions. The remaining three shifted SSCP bands were found to constitute missense mutations: G140C (Ser47Thr) in exon 1, A1591G (Lys531Glu) in exon 8, and T1609A (Tyr537Asn) in exon 8. While the former two sequence changes result in substitution of residues within hydrophilic amino acid groups, the later mutation changes a hydrophobic tyrosine to a hydrophilic asparagine.

It has been estimated that missense mutations are present in only about 1% (2/188) of primary tumors (4). In agreement with this, we have not detected any missense ER alterations in 60 primary breast cancers that we have examined using SSCP analysis (unpublished data). In this study of 30 cases of metastatic, Tam-resistant breast cancer, we detected three missense ER mutations. Thus ER mutations in metastatic breast tumors may be more frequent as compared to that seen in primary lesions (10% as compared to 1%,  $p=0.004$  with Fisher's exact test).

### **Specific Aim 2**

The functional transactivational status of the three missense ER mutations isolated from the metastatic breast tumors were first investigated using transient transactivation assays, measuring the transcriptional activity of the mutant ERs with ERE-reporter gene constructs. Since the majority of studies examining the effect of specific alterations in the ER on function have utilized consensus EREs, most commonly the vitellogenin A2 ERE, we felt that it would be important to test ER function on constructs which might be more relevant to breast cancer biology, eg. those genes which are endogenously regulated by ER in breast cancer cells. We thus prepared ERE-CAT reporters to the estrogen-regulated pS2, Cathepsin D, and lactoferrin gene promoters. Each mutant ER was then cotransfected into HeLa cells (Fig. 1) with the different ERE reporter vectors and compared to wtER activity. CAT activity was determined relative to the activity of the

reporter vector-alone transfected into cells; activity was also corrected for transfection efficiency by cotransfection of a  $\beta$ -gal vector. The activity of Ser47Thr and Lys531Glu were not different from that of wtER using any of the four different ERE constructs. However, the Tyr537Asn mutant exhibited strong constitutive transactivation activity (15-20-fold over wt ER activity on the vitellogenin ERE) in the absence of hormone (Fig.1, vitellogenin con). This elevated constitutive activity was also observed on the other ERE constructs (5-fold on the pS2 con, 8-fold on the Cathepsin D con, and 17-fold on the lactoferrin ERE con). Estradiol was required for the induction of wtER activity on all four of the ERE reporter constructs (maximum inductions were 11-, 2-, 3-, and 4-fold on the vitellogenin, pS2, Cathepsin D, and lactoferrin reporters, respectively, using  $10^{-9}$  M estradiol; Fig. 1). As expected, tamoxifen alone had no effect on basal activity of the wt ER, and completely inhibited the stimulatory effect of estradiol (Fig. 1, E2 + Tam).

In contrast, the addition of estradiol had only minimal influence on the already high constitutive transcriptional activity of the Tyr537Asn mutant in HeLa cells (Fig. 1, compare the con vs the E2-stimulated levels). Interestingly, tamoxifen appeared to slightly inhibit to varying degrees the basal activity of the Tyr537Asp mutant on three of the ERE promoters; for instance, 45% inhibition of basal activity was seen using the Cathepsin D ERE reporter. The only notable difference among the four ERE reporters was in the insignificant tamoxifen inhibition of basal transcriptional activity on the lactoferrin promoter (Fig. 1, compare con vs tam levels). Similar results as that seen with tamoxifen were observed when these experiments were repeated using the pure antiestrogen ICI 164,384 in these cells (data not shown).

We know that the transcriptional activity of the ER is highly dependent on the cell and the promoter context (5) in which the receptor is expressed, thus we also tested the transcriptional activity of the Tyr537Asn ER in MDA-MB-231 breast cancer cells. Similar

results were seen in these cells. Again, the Try537Asn mutant displayed high constitutive activity on all four of the ERE reporters, ranging from 3 to 7-fold depending on the ERE reporter, and this activity was essentially unaffected by estrogen, tamoxifen, or the ICI pure antiestrogen (data not shown).

The Tyr537Asn mutation eliminates a tyrosine residue which is a potential phosphorylation site within the ER. Several potential mechanisms could explain the high constitutive activity of the tyr537asp ER mutation. One explanation, and one that we favor, is that the Tyr537Asn substitution may produce a conformational change in the receptor that mimics hormone binding. We know that this residue lies within the hormone binding domain of the ER; functional analysis of ER deletion mutants has suggested that the carboxy-terminal boundary of both the estrogen and tamoxifen-binding domains are similar, lying between residues 522 and 538 (6, 7). The role of ligand binding in the formation of AF-2 is unknown, but is believed to involve conformational changes in the receptor which generates a productive association between the AF-1 and AF-2 domains (8). We envision that similar conformational changes may be induced by the Tyr537Asn substitution. As a result of this conformational change, the mutant ER might only weakly bind estrogen and tamoxifen, explaining their limited effects on mutant ER activity. Accordingly, there is evidence to suggest that phosphorylation at this site is required for efficient estrogen binding. Notides and coworkers (9) determined that tyr 537 is a physiological phosphorylation site in ER isolated from MCF-7 human breast cancer cells. Using site-directed mutagenesis to separately replace all five tyrosine residues within the hormone binding domain of the ER with phenylalanine, Castoria et al. (10) demonstrated that phosphorylation of *in vitro* synthesized ER at tyr537 confers efficient estrogen binding ability. Thus, phosphorylation of Tyr537 may induce conformational changes that are necessary for ligand binding.

It has also been suggested that phosphorylation at tyr537 is a necessary step for ER dimerization (11). These authors propose that ER dimerization occurs through specific interactions between phosphotyrosine residues and src-homology (SH2)-like domains, similar to the activation of the STAT family of transcription factors (12). This data would therefore suggest that the Tyr537Asn ER mutant would be incapable of dimerization due to the absence of a target for the SH-2 like domain of its dimerization partner. However, as ER dimerization is necessary for ERE binding and transcriptional activity, this hypothesis is clearly inconsistent with our demonstration of strong transcriptional activity with the Tyr537Asn mutant, and implies that other regions of the hormone binding domain, such as the adjacent leucine zipper motif, are involved in dimerization as well.

Tyr537 may represent a basal phosphorylation site of the human ER which is under strict control by both specific tyrosine kinases and phosphatases. The Tyr537Asn ER mutant may have escaped from phosphorylation-mediated transcriptional regulation that is present *in vivo*, as was seen in the HeLa and MDA-MB-231 cells used in the present transfection studies. Disruption or dysregulation of phosphorylation at specific sites within the ER may therefore be important in the clinical problem of hormone-independent tumor growth, as would be predicted for patients harboring the Tyr537Asn mutation detected in this study. Future studies will be directed at investigating the frequency of this specific alteration in patients with metastatic breast cancer to determine whether this constitutive mutation is common in patients with dissemination of their disease. In addition, we will be evaluating the Tyr537Asn mutant in stable transfectants both *in vitro* and *in vivo* for properties such as tamoxifen-resistant growth, and are currently generating a Flag-tag expression vector (Eastman Kodak Co.) for generating the transflectants. In addition, we plan to use the reverse tet-inducible expression system for these studies. These studies will proceed into the third and be completed by the last year of the grant proposal. The results

summarized above have been submitted to Cancer Research (An estrogen receptor mutant with strong hormone-independent activity from a metastatic breast cancer; Qui-Xia Zhang, Ake Borg, Douglas Wolf, Steffi Oesterreich, and Suzanne Fuqua).

### **Specific Aims 3 and 4**

The second part of this research fellowship proposal (Specific Aims 3 and 4) involves the identification of new genes potentially involved in the development of Tam resistance. This was to be accomplished using the technique of differential display (DD) analysis of RNA isolated from Tam-sensitive vs. Tam-resistant tumors. We found in the first year of the grant that DD was not well suited for these tumors because they are composed of many different cell types, and thus we have spent the second year of the proposal addressing this technical issue. The results of DD from whole tumors are simply uninterpretable unless the RNA is prepared from a highly purified sample of one cell type. To this aim, we have now developed methodologies for microdissection from frozen and paraffin-embedded, archival breast tumor tissues. Microdissection is quite difficult on unstained slides, which motivated us to first do a study comparing various histochemical stains to find one that provided adequate visualization for microdissection without interfering with RNA isolation. Our results (Fig. 2) showed that nuclear-fast-red (NFR) satisfied these requirements, and we now use NFR routinely for all microdissections. We found that during microdissection, specific cells of interest could be visualized by staining briefly with NFR and quickly redrying the slide, allowing for better direct visualization and only slightly decreasing the yield of RNA. Accurately separating different types of cells obviously requires familiarity with the histopathological features of the tissue, and Dr. Craig Allred, a Breast Pathologist at this Institution and a member of the San Antonio Breast Cancer Research Team, is collaborating with us on this aspect of the project.

We also found that DD was best performed using RNA isolated from microdissected frozen, or air-dried frozen-section samples (Fig. 3). DD performed on RNAs isolated from routine clinical paraffin-embedded sections appears to be unreliable, thus making interpretation difficult (although RNA from tissues rapidly fixed in formalin for short periods of time can be of high quality). The results summarized in Aim 3 have recently been published (Castles, C.G., Allred, D.C., Krieg, S.L., Benedix and Fuqua, S.A.W.: RNA from air-dried frozen sections for RT-PCR and differential display. BioTechniques 21:425, 1996). Thus we now feel that we have the methodologies worked out to begin the DD analysis of the breast tumor samples as outlined in Specific Aims 3 and 4 of the grant.

## CONCLUSIONS:

We have successfully completed Specific Aim 1 and will begin the last part of Aim 2 (stable transfection of the Tyr537Asn and analysis of *in vivo* growth characteristics). Thus we will complete the first half of the proposal as originally planned, on schedule. Our conclusion is that although mutations in the ER are rare in primary breast cancer, they may be more frequent (3/10) in metastatic breast lesions. We will also determine whether the one ER variant we identified, the Tyr537Asn, which exhibits a phenotype consistent with tamoxifen-resistance, is expressed in other breast tumors and may thus play a role in the problem of clinical tamoxifen resistance.

It has become apparent that other ER-associated factors, called receptor interactive or accessory proteins, may play an important role in modulating ER function, and thus may play a role in the agonist acitivity of tamoxifen. We might be able to identify some of these factors if they are differentially expressed in Tam-resistant tumors using the technique of DD. An alternative method, such as yeast two hybrid assay, may be one another assay

that we will employ in the final phases of this project, in addition to DD analysis, to address this new possibility. We think that we are uniquely positioned to attack the clinical problem of Tam resistance with our extensive tumor bank and our recent experience in microdissection and harvesting of RNA adequate for these techniques.

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**FIGURE LEGENDS:**

**Figure 1:**

The Tyr537Asn ER mutant displays strong hormone-independent transcriptional activity in HeLa cells. Transactivation assay comparing the Tyr537Asn ER mutant (light columns) with wt ER (black columns) in the absence and presence of estradiol (E2,  $10^{-11}$  and  $10^{-9}$  M), 4-hydroxytamoxifen (Tam,  $10^{-7}$  M), and a combination of both (E2  $10^{-9}$  M and Tam,  $10^{-7}$  M). The results from four different ERE constructs are shown in separate panels. Bars represent % CAT conversion (corrected for  $\beta$ -gal activity) from duplicate wells  $\pm$  S.D.

**Figure 2:**

RT-PCR analysis of RNAs isolated from archival sections of normal breast. The left panel is a PCR amplification of beta-2-microglobulin (185 bp) using unstained (U), giemsa (G), methyl green (MG), nuclear fast red (NFR), eosin (E), and hematoxylin (H) staining prior to microdissection. NFR was nearly equivalent to unstained slides, and slightly better than MG or G. The two most commonly used histochemical stains, H and E, both interfered with the signal. The right panel shows an amplification of beta-actin (A; 270 bp) and the estrogen receptor (ER; 200 bp) from microdissected archival normal breast tissue stained with NFR. The size markers (M) are shown on the left of each panel.

**Fig. 3:**

DD results from air-dried frozen and permanent (perm.) sections. Reverse-transcribed cDNA was amplified by PCR from histological sections of endocervix using a T11CA antisense primer and a random 10-mer sense primer as described in the text. In Figure 2a, the large number of bands in the air-dried frozen samples (lanes 3 and 4) demonstrates that DDRT-PCR can be used for analysis of RNA species as large as 600

base pairs extracted from tissues of this type. Tissue from the sample in lane 3 was stained with hematoxylin. MCF-7 cell RNA (lane 1) and RNA from a frozen section of endocervix (lane 2) were used as positive controls. In Figure 2b, DD was performed on RNA recovered from formalin-fixed permanent sections of endocervix with (+) or without (-) hematoxylin staining. Negative controls lacking RT generated no significant banding patterns (not shown). M=*MspI*-digested pBR322 plasmid (Promega, Madison, WI).

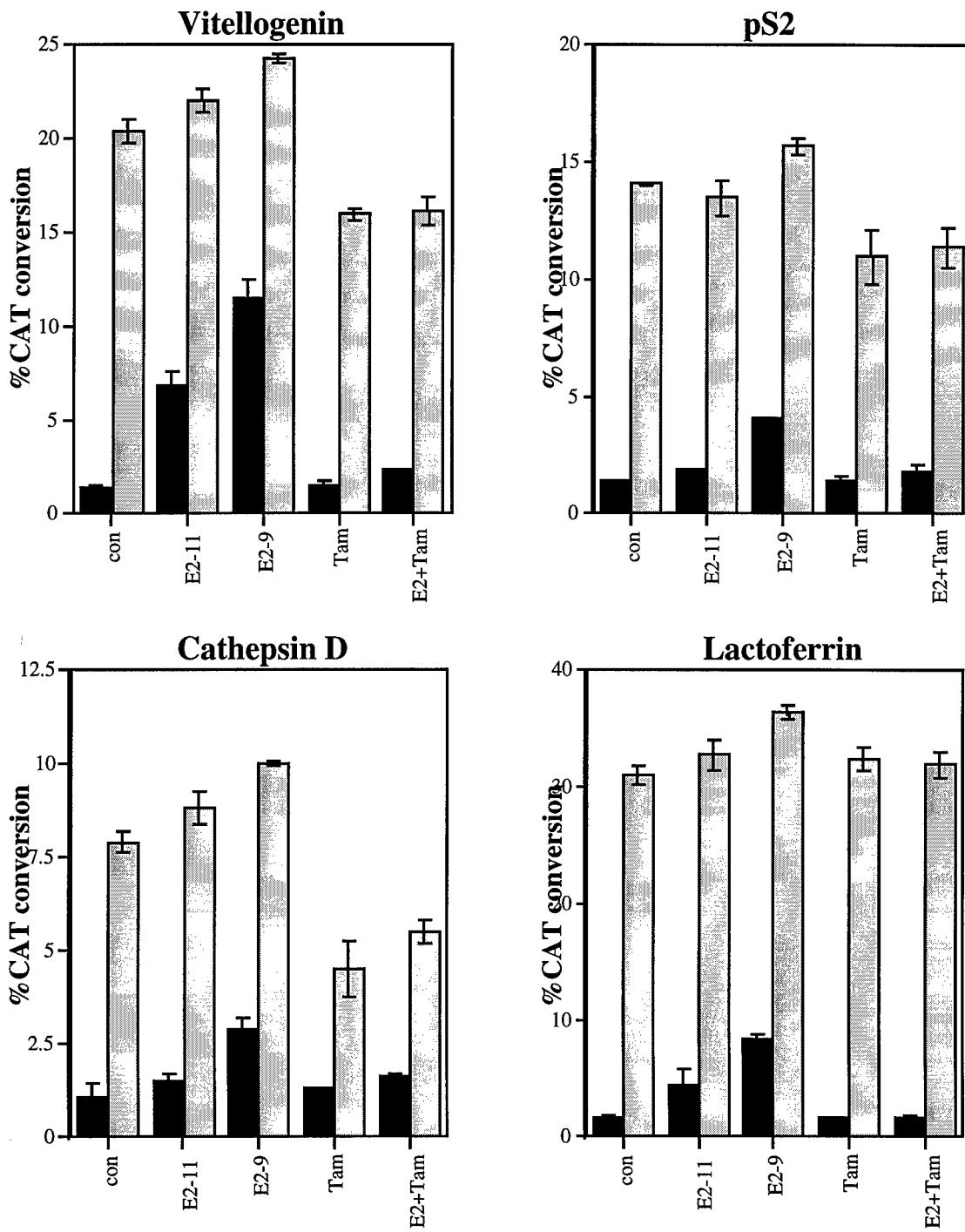


Figure 1

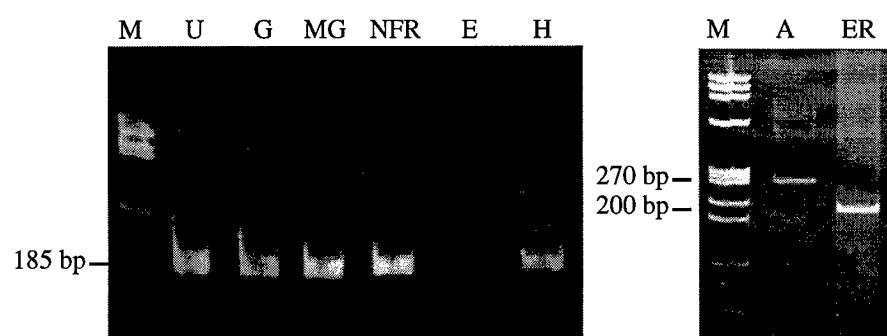


Figure 2

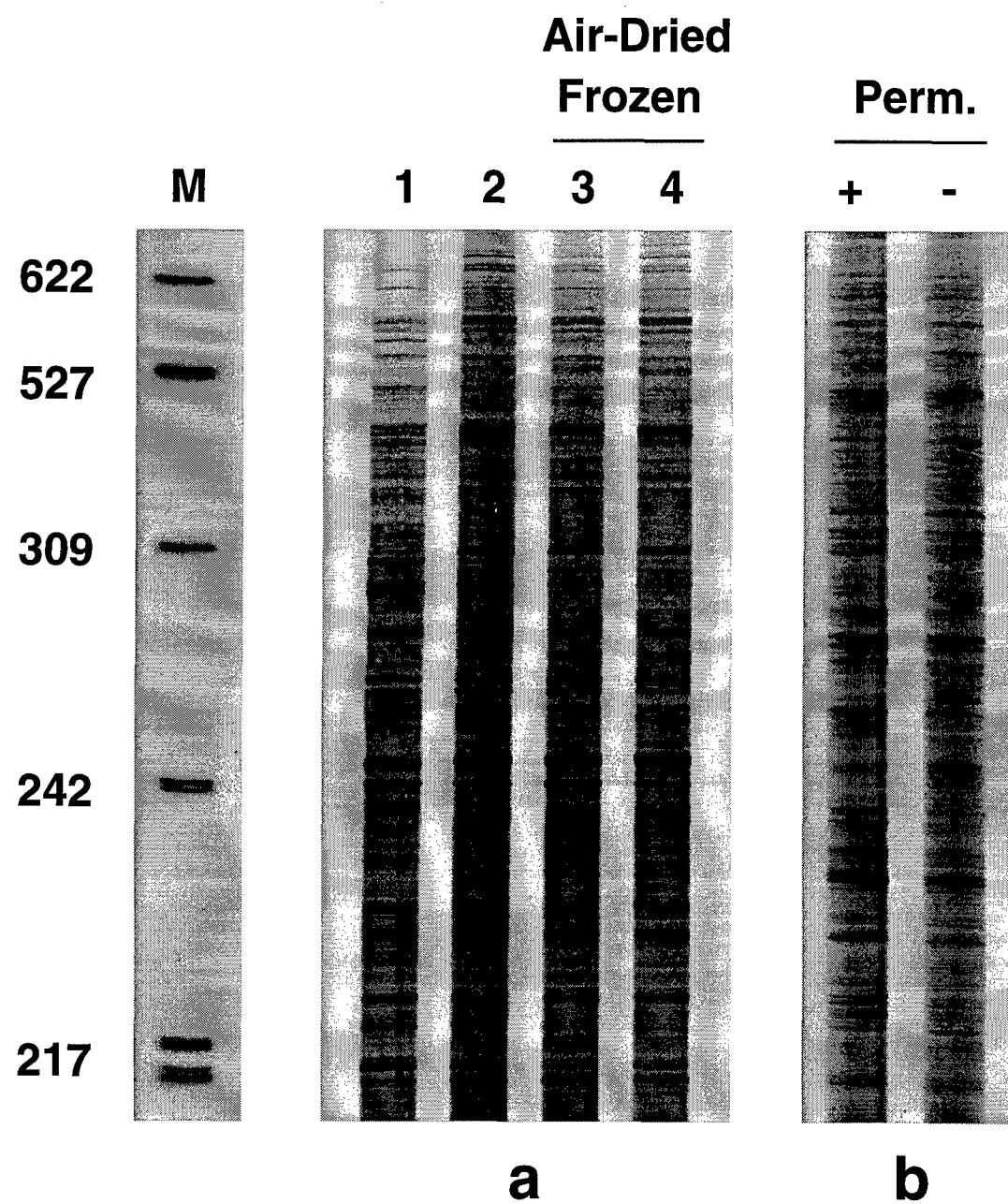


Figure 3



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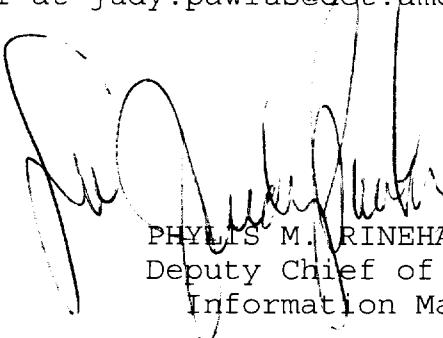
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